5.1 Parasitology Introduction

The following chapter describes identification procedures for four parasitic infections of fish that are commonly included in a fish health inspection. The target parasite species include three myxozoan parasites of salmonid fishes: *Myxobolus cerebralis, Ceratomyxa Shasta, and Tetracapsula bryosalmonae*; and the cestode: *Bothriocephalus acheilognathi,* which infects members of the Family Cyprinidae. Chapter 2 Sampling describes procedures for proper sampling of fish tissues to ensure detection of any of these pathogens during a fish health inspection.

For *Myxobolus cerebralis*, presumptive identification is based on identification of the myxozoan spore stage from pepsin-trypsin digested (PTD) cartilage. Tissues from up to five fish may be pooled for screening by PTD. Identification of the spores is based on morphology. Confirmatory identification is based on identification of the spores in histological sections or on amplification of *M. cerebralis* DNA by the polymerase chain reaction.

For *Ceratomyxa shasta*, presumptive identification is based on identification of myxozoan spore or trophozoite/presporogonic stages from intestinal tissue. Identification of the myxospore stage is sufficient for confirmation of infection. Identification of the earlier stages must be confirmed by amplification of *C. shasta* DNA by the polymerase chain reaction.

For *Tetracapsula bryosalmonae*, presumptive identification is based on identification of the presporogonic stages of the parasite in Leishman-Giemsa or lectin stained imprints of kidney or spleen tissue. Infection is confirmed by identification of these stages in histological sections of kidney tissue or on amplification of *T. bryosalmonae* DNA by the polymerase chain reaction.

For the cestode, *Bothriocephalus acheilognathi,* visualization of any cestode with a pyramidal scolex results in a presumptive positive classification. Confirmation requires verification of morphological characteristics of the scolex.

DISCLAIMER: Mention of specific brands or manufacturers does not warrant endorsement by the U. S. Fish and Wildlife Service, the United States government, and /or the American Fisheries Society. Any comparable instrument, laboratory supply, or reagent may be substituted in this protocol if operation and performance are deemed comparable to the items specified.

5.2 *Myxobolus cerebralis* (Whirling Disease)

Myxobolus cerebralis may be difficult to detect because the life cycle of the parasite includes two alternate hosts: salmonids and the aquatic oligochaete worm, *Tubifex tubifex* (Wolf and Markiw 1984); and the extended time required for sporogenesis in the salmonid host. For an *M. cerebralis* inspection, sampling should target the most appropriate species, age, and rearing units that are most likely to reflect the presence of the parasite. The species most susceptible are rainbow trout, sockeye salmon, and steelhead. Brook trout, cutthroat trout, Atlantic salmon, and chinook salmon are moderately susceptible; brown and bull trout, coho salmon, and splake are partially resistant. Conflicting data are present for Arctic grayling and lake trout but in general, these species may be considered resistant or partially resistant to *M. cerebralis.* Fish are most susceptible if exposed when young; however, older fish may become infected and act as carriers of spores. Fish that have a high degree of resistance should not be selected for sampling unless they are the only species present (O'Grodnick 1979; Hedrick et al. 1999a, b; Hedrick et al. 2001; MacConnell and Vincent 2002).

Development of myxospores is temperature dependent, requiring a minimum of 90 days at 16 to 17˚C, and 120 days at 12 to 13˚C (El-Matbouli et al. 1992). When temperature data is available, sample fish that have been on the water supply for a minimum of 1800 degree-days. If continuous temperature monitoring data is not available, select fish that have been on the water supply for a minimum of six months. Select fish that reside in locations on an aquaculture facility most likely to result in exposure to the parasite such as earthen rearing containers and from locations receiving untreated surface water.

Screening for *M. cerebralis* is by examination for spores in cranial cartilage processed by pepsin-trypsin digest. Up to five fish may be pooled for screening; confirmation is on individual fish. Subsampling from large fish by using a halved head or a core sample is accepted; however, detection sensitivity may be decreased if the number of cartilaginous areas containing the parasite are few or if parasite numbers are low. Another difficulty is parasite affinity for different tissue sites may vary between salmonid species (in rainbow trout the parasite prefers the ventral calvarium; and brown trout the parasite is detected in gill arches). Confirmation of *M. cerebralis* is by identification of parasite stages in histological sections of cartilage tissue, or by amplification of parasite DNA by the polymerase chain reaction (PCR).

A. Screening Test

1. **Pepsin-Trypsin Digestion (PTD)** (Markiw and Wolf 1974; Lorz and Amandi 1994)
   This method is recommended for fresh samples; if the sample has been frozen, follow the procedure below with modifications noted in A.1.c.1 and A.1.d.

   a. Defleshing

      i. Samples should be placed in an appropriate container and heated at 45°C in a water bath until flesh is soft and eyes are opaque.

      ii. Deflesh the samples and retain all cartilage/bone including that from the gill arches and opercula.

   b. Pepsin Digestion

i. Weigh cartilage and add 0.5% pepsin solution (5.6.A “0.5% Pepsin Solution”) at a ratio of 20 mL/g cartilage.

ii. Large heads (greater than 20 g after defleshing) may be homogenized in the pepsin solution with an electric blender.

iii. Stir at 37°C and monitor pH of pepsin solution. If pH increases above 4.0, centrifuge samples at 1200 x g for 10 minutes, decant pepsin, and add fresh pepsin to the sample.

iv. Process samples until all cartilage/bone is reduced to a consistently small, granular size (e.g. the size of beach sand; one hour is generally sufficient for small fish; two hours to overnight may be required for larger fish).

v. When digestion is complete, centrifuge at 1200 x g and decant pepsin into a solution of 1:4 bleach/water (5,000 ppm chlorine) for disposal.

c. Trypsin Digestion

i. Add 0.5% trypsin (5.6.B “0.5% Trypsin Solution”) at a ratio of 20:1 (volume/g) to the pellet.

Note: If samples have been frozen prior to processing, reduce the concentration of trypsin to 0.05%

ii. Adjust to pH 8.5 with 1 N sodium hydroxide (5.6.C “1N Sodium Hydroxide (NaOH)”).

iii. Stir at room temperature for 30 minutes.

iv. If samples are to be refrigerated at this stage, inactivate trypsin by adding serum to a final concentration of 20%, or powdered bovine serum albumin to a final concentration of 1%.

v. Pour digested sample through a non-absorbent disposable filter [e.g. paint filters (any paint store), urinary calculi filters, or synthetic material such as nylon screen] into a new tube. Mesh sizes should approximate 200 µm. Reusable filters are not recommended because of difficulties in decontamination. Autoclave filters before disposal.

vi. Centrifuge at 1200 x g for 10 minutes.

vii. Discard supernatant into a solution of 1:4 bleach/water (5,000 ppm chlorine) while retaining pelleted material.

d. Dextrose Centrifugation (Markiw and Wolf 1974)
This concentration step is recommended if spores are not found on initial examination or if a large amount of tissue debris makes microscopic examination difficult; it is not recommended if samples have been frozen because it causes distortion and decreased recovery of the spores.

i. Add 1 mL of phosphate buffered saline (PBS) (5.6.D “Phosphate Buffered Saline (PBS)”) to the sample and vortex (this volume may need to be adjusted depending on pellet size, but should be sufficient to suspended all material).

ii. A 55% solution of dextrose is used to provide a 5 cm deep gradient in a 15 mL centrifuge tube. For larger samples, a 50 mL centrifuge tube may be required.

iii. Carefully layer the suspended sample onto the dextrose solution.

iv. Centrifuge at 1200 x g for 30 minutes.

v. Carefully decant the supernatant into a solution of 1:4 bleach/water (5,000 ppm chlorine) while retaining all pelleted material.

vi. If PTD sample will be used for PCR confirmation, the pellet should either be suspended in 0.5 to 1.0 mL of PBS and examined immediately (a 10 fold dilution of the pellet should be sufficient to disperse material enough to see clearly under the microscope). The sample may then frozen until assay or preserved using 70% ETOH.

e. Analyzing Samples

i. Vortex samples prior to preparing slide.

ii. Samples may be examined stained or unstained by wet mount or using a hemocytometer. A number of simple staining methods are suitable for staining spores; e.g. add 60 µL of a saturated solution of crystal violet biological stain to 1 mL subsample, mix and examine.

iii. A minimum of 150 fields should be examined at 200x magnification (20x objective with 10x ocular eyepiece). Measure ten representative myxospores and record the average size and size range. Identification of myxospores with the appropriate size and morphology (8 to 10 µm, rounded, two polar capsules; Figure 5.1) results in a sample designation of **PRESUMPTIVE positive.** If the size is appropriate but internal morphology is not clear, the sample should also be identified as **PRESUMPTIVE positive.** A single myxospore of appropriate size is sufficient to declare a **PRESUMPTIVE positive**. **All other samples are reported as negative.**

Note: Other myxobolid species of salmon have similar morphologies that may be confused with *M. cerebralis*. Photographs and measurements of some of these species are provided in Figure 5.2 for comparison.

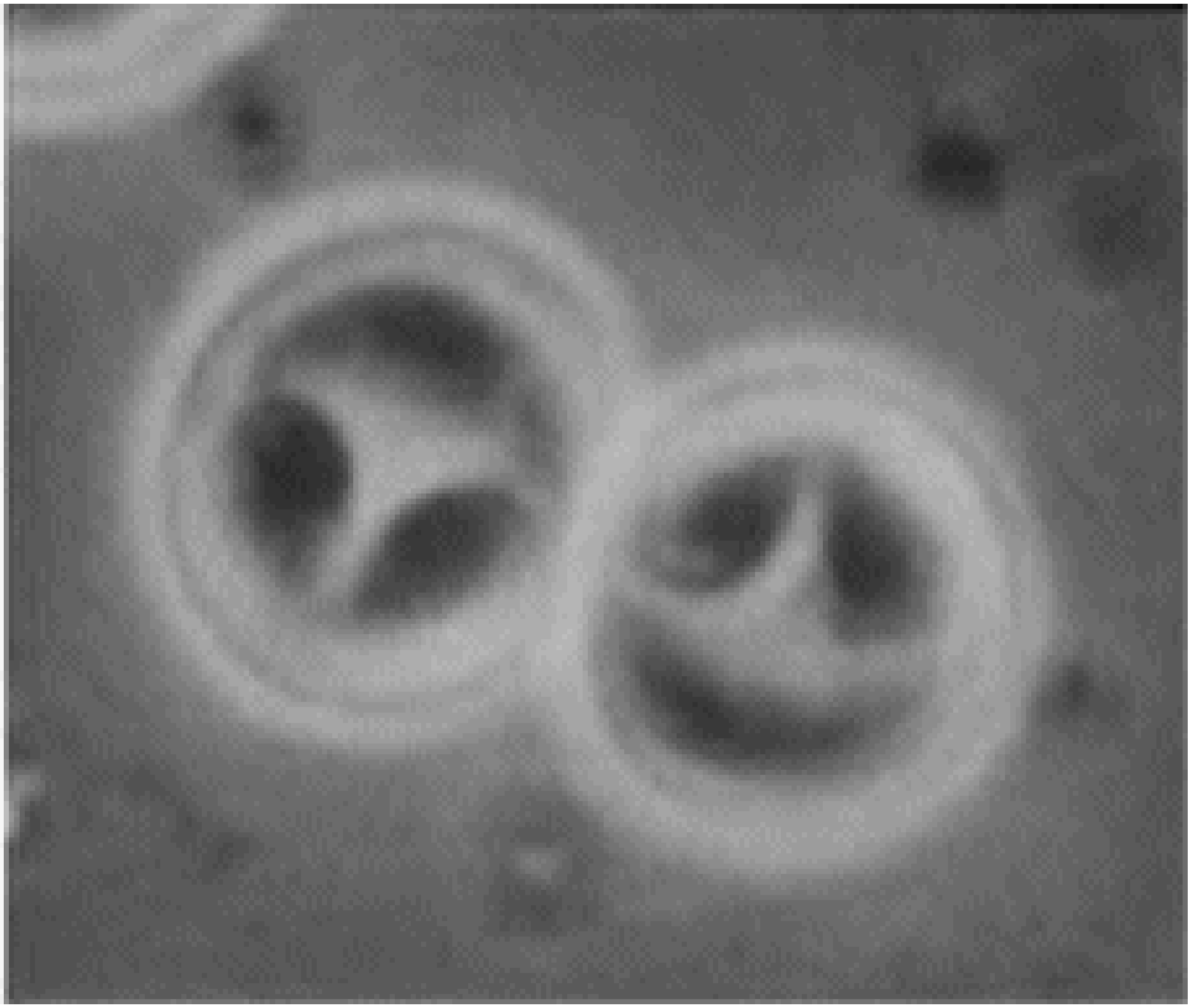
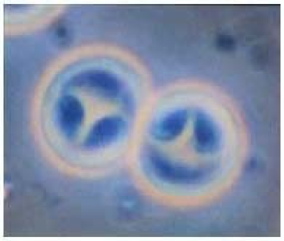


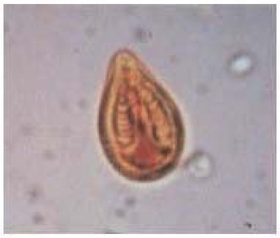
Figure 5.1. Spores of *Myxobolus cerebralis.* Photograph courtesy of R. P. Hedrick.

Figure 5.2: Myxozoans Common To Salmonid Fish



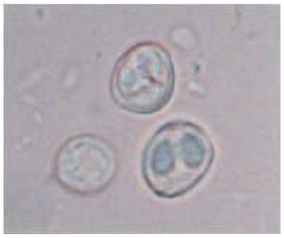
Myxobolus cerebralis

L 7.4 - 9.7 x W 7 - 10 x T 6.2 x 7.4 μm



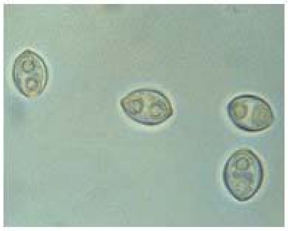
Myxobolus insidiosus

L 12.8 - 17.1 x W 8 - 11.9 μm



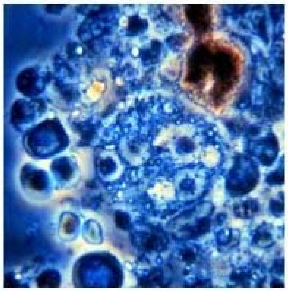
Myxobolus neurobius

L 10 - 14 x W 8 - 9.2 μm

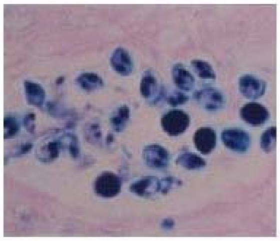


Myxidium minteri

L 9.3 - 12.6 x W 4.5 - 5.3 μm

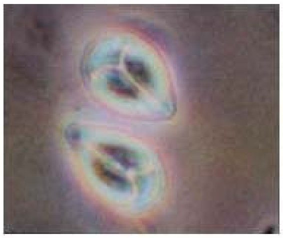


PKX organism



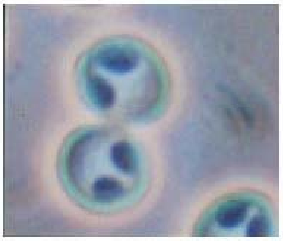
Myxobolus kisutchi

L 8.5 x W 7 μm



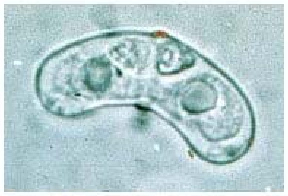
Myxobolus arcticus

L 14.3 - 16.5 μm.



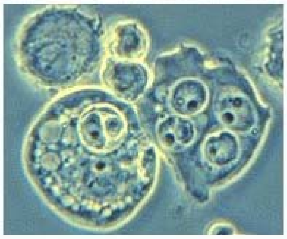
Myxobolus squamalis

L 8.4 - 9.9 x W 7.7 - 9.9 μm



Ceratomyxa shasta

L 14 - 23 x W 6 - 8 μm



Ceratomyxa shasta trophozoites

B. Confirmatory Tests

Confirmation of *M. cerebralis* should rely on either histopathology or the nested version of the polymerase chain reaction (PCR).

1. Histopathology

a. Confirmation of *M. cerebralis* may be done using standard histological techniques. Detection of spores with the morphological characteristics of those identified in the digest preparation in tissue other than the cartilage (e.g. brain, nervous tissue, skin, or muscle) is sufficient to report the sample as negative for *M. cerebralis*. However, failure to detect spores of the correct morphology in any tissue is not sufficient to report the sample as negative. Half heads, wedges, or core samples, which were placed in 10% neutral buffered formalin or Davidson's fixative at the time of collection (2.2.E.4 "Collection of Specimens for the Detection of *Myxobolus cerebralis* Evaluation") will be used for corroboration. Process tissues corresponding to a sample number in which a presumptive positive was found.

b. Small samples (from fish <15 cm) fixed in Davidson's can be transferred to 70% ethanol after 24 to 48 hours. Larger samples should remain in Davidson's for 48 hours and may require an additional decalcification step (as specified below).

c. Samples fixed in 10% formalin should be transferred after 24 to 48 hours to a commercially available chemical decalcification solution (e.g. Lerner-D-Calcifier, Decal-Stat Solution, Cal-Ex, and others) or acid decalcifying solution (e.g. 10% Nitric Acid, 5% Formic Acid).

d. Samples requiring decalcification are placed into embedding cassettes in a beaker with a magnetic stir bar and sufficient decalcifying solution (20:1 solution to gram of head tissue). Covered and place on a stirring plate for 4 to 16 hours at room temperature. After three hours, and periodically thereafter, use physical and/or chemical tests to determine if the process is complete (Hauck and Landin 1997).

   i. Physical
   Probe using a dissecting needle to determine softness of bone and/or cartilage (avoid puncturing the tissues). Soft tissues are adequately decalcified.

   ii. Chemical
   Remove 5 mL of decalcifying solution from beaker and neutralize (pH 7.0) with 0.1 N sodium hydroxide (5.6.C "1N Sodium Hydroxide (NaOH)" stock solution). Add 1 mL of saturated ammonium oxalate solution and mix. Allow to stand for 15 minutes and determine if a precipitate (slight cloudiness) of calcium oxalate forms. If so, the decalcification is incomplete. If incomplete, replace decalcifying solution and continue decalcification process until no calcium oxalate precipitate can be detected.

   iii. After decalcification, rinse specimens in distilled water and transfer via graded ethanol series to 70% ethanol.

e. Embed and section tissues at approximately 4 to 5 µm using standard methods. Two sections, taken at 100 µm apart, should be mounted from each sample (or each tissue block if more than one block per fish) and stained with Giemsa (May-Grunwald Giemsa works well, Luna 1968) or Hematoxylin and Eosin (H&E). The sections should target gill arches (especially from brown trout) and the ventral calverium (floor of the brain case). Examine slide at 200X for cartilage lesions, spores and developing stages of *M. cerebralis* (Figures 5.3 and 5.4).

f. At least two additional sections (100 µm apart) should be taken, mounted and stained if histological lesions and/or spores are not observed.

Observation of any presporogonic or sporogonic stage of *M. cerebralis* in cartilage tissue is reported as positive. Observation of spores with the same morphology as those identified in the digest preparation in tissues other than cartilage are reported as negative. Confirmation cannot be reported if no spores are detected.

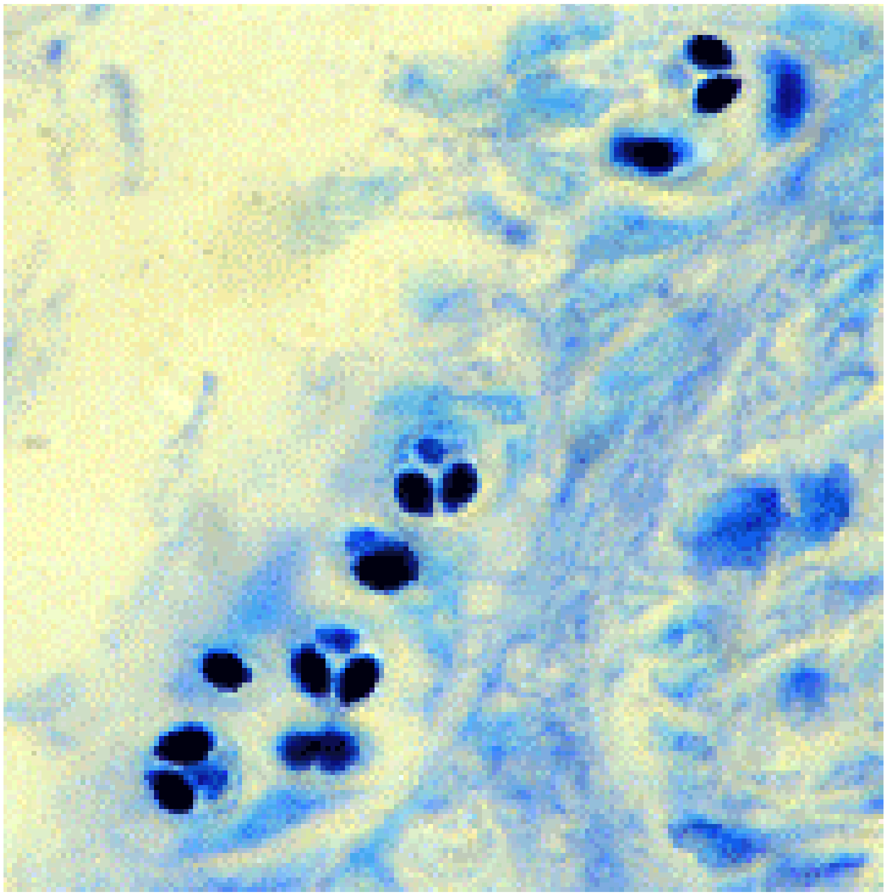


Figure 5.3. Histological sections from a fish infected with *Myxobolus cerebralis*. High magnification showing stained spores (note darkly stained polar capsules). Photograph courtesy of H. V. Lorz.

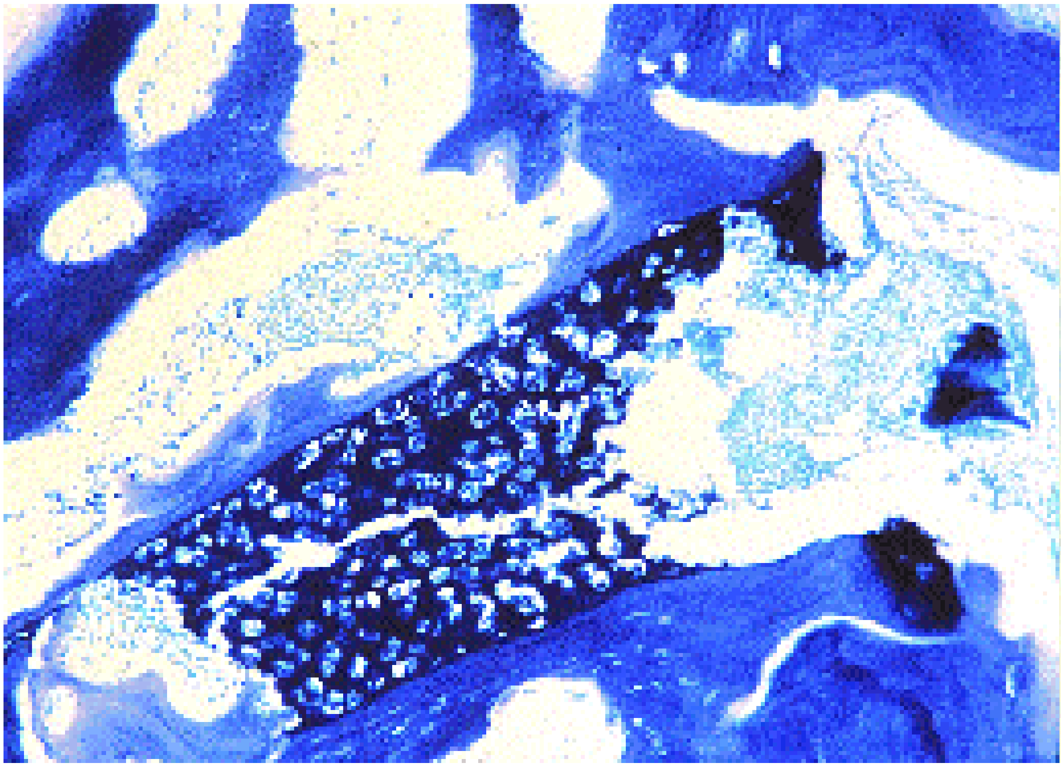


Figure 5.4. Histological sections from a fish infected with *Myxobolus cerebralis*. Low magnification showing degradation of cartilage (Leishman-Giemsa stain).

2. **Polymerase Chain Reaction (PCR)** (Andree et al. 1998; Schisler et. al. 2001)
PCR should be utilized as a confirmatory test *only* on tissue collected as described under specimen collection for PCR at the time of inspection. The assay may be performed directly on the remaining half head or core sample, allowing detection of target DNA from immature parasites as well as from spores (5.2.B.2.a "Extraction of DNA from a Half Head or Core for PCR Confirmation of *Myxobolus cerebralis*"). An alternative method (Baldwin and Myklebust,2002) identifies *M. cerebralis* DNA from spores concentrated by PTD (5.2.B.2.b "Extraction of DNA of the PTD Product for PCR Confirmation of *Myxobolus cerebralis*"). Use of this method allows the other half head to be preserved for histology. However, by applying PCR methods to a digested preparation DNA, presporogonic life stages of the parasite are destroyed, with a potential loss of detection sensitivity. The University of California has awarded an exclusive license to Biogenetic Services, Inc., for the use of the DNA-based diagnostic test for detecting the *Myxobolus cerebralis* organism described by Andree et al. (1998). Contact Biogenetic Services, Inc. for additional information on the availability and limitations to the use of this PCR-based technology. (Phone 605/697-8500; Fax 605/697-8507; Email: biogene@brookings.net; Website: www.biogeneticservices.com)

    a. Extraction of DNA from a Half Head or Core for PCR Confirmation of *Myxobolus cerebralis*

    Note: The following protocol recommends use of the Qiagen DNeasy Tissue Kit (Qiagen Inc, Valencia, CA; Qiagen #69506). Although other kits may work similarly, they have not been tested for this application. Use the kit as per the handbook, with the following modifications:

i. See 6.2 PCR – Quality Assurance/Quality Control for specific QA/QC considerations for PCR.

ii. Place half-head or core sample in screw capped tubes appropriate for the sample size. Add tap water to cover the tissue and heat in a water bath at 95°C for 15 minutes. For heads ≥ 5 centimeters (2 inches), use 8 ounce sample cup or small beaker and heat for 20 minutes.

iii. Pour water into a solution of 1:4 bleach/water (5,000 ppm chlorine) for 30 minutes to disinfect and place fish sample on polypropylene cutting mat or other surface that can be disinfected or disposed of (autoclave).

iv. Deflesh head using a clean scalpel and forceps; place bone and cartilage in a 1.7 mL micro centrifuge tube or a 50 mL polypropylene screw cap centrifuge tube for larger samples.

v. Add a sufficient volume of ATL buffer, such that it is mixed with the bone material approximately 1:1 (v:w) and proteinase K solution equivalent to 1/10 volume of the buffer. If glass beads are used, extra beads can be added for the larger heads.

Tissue lysis buffer	ATL buffer (Qiagen #69504)
Proteinase K solution (See 5.6.E "Proteinase K.")	(17.86 mg/mL)
150 mg small glass beads (optional)	710 – 1180 µm, acid washed
3 large glass beads (optional)	3 mm, acid washed

vi. Vortex until the sample is broken up.

vii. Incubate at 55°C on a rotating platform (or with occasional vortexing) for at least 3 to 4 hours to overnight.

viii. Centrifuge at 16,000 x g for five minutes.

ix. Add 200 µL *aqueous* supernatant to clean micro centrifuge tube.

Note: Occasional clear layer above the white lipid layer is oil; aqueous layer is between lipid layer and debris/glass bead pellet and may be turbid. Dark particulates do not affect DNA yield but can increase column spin times required.

x. Add 20 µL RNase A (5.6.F "RNAse A") (20 mg/mL; Qiagen #19101), vortex until mixed thoroughly, and incubate at room temperature for two minutes.

xi. Follow kit instructions for elution of DNA using buffers and spin column supplied in the kit (DNeasy Tissue Kit; Qiagen #69506), with the following precautions:

Note: "Dirty" preps may require longer spins. DNA preps from positive samples give consistent results when using spin columns. When expecting very small amounts of DNA, the volume of elution buffer can be reduced to 50 µL.

b. Extraction of DNA of the PTD Product for PCR Confirmation of *Myxobolus cerebralis*

i. Pellet the myxospores of the PTD product by micro centrifugation at maximum speed (at least 10,000 rpm) for one minute.

ii. Decant the supernatant and air dry for five minutes

iii. Microwave for one minute in an 800-watt microwave oven at full power.

iv. Re-suspend the pellet in 500 µL of Proteinase K lysis buffer (10 mM Tris-Cl, pH 8.0, 2 mM EDTA, 0.1 % sodium dodecylsulfate and 0.5 mg mL^{-1} Proteinase K) and incubate at 55°C for four hours frequently vortexing gently.

v. Remove sample to a clean laboratory hood and add 500 µL of phenol:chloroform:isoamyl alcohol (25:24:1) to the lysed sample.

Note: Procedures are being developed to substitute the phenol:chloroform extraction (steps v–x) with extraction using Qiagen Kits (Qiagen Inc, Valencia, CA, Qiagen # 69506). Currently this work remains unpublished, but the procedure is as above, with the modification that after microwave treatment, 180 µL of Qiagen Buffer ATL and 20 µL of Proteinase K are added to the sample. The sample is vortexed and incubated at 55°C for 6 to 8 hours for complete cellular lysis. The DNA then can be selectively bonded to the Dneasy membrane columns, washed to reduce contaminates and inhibitors, then eluted using the Dneasy protocol for rodent tails. As will any procedure modifications, this should be thoroughly tested and documented in the laboratory.

vi. Gently invert sample several times to form an emulsion, then centrifuge at 1700 x g at room temperature for 10 minutes to separate the aqueous and organic phases.

vii. Transfer the upper aqueous phase (with the nucleic acid) to a clean micro centrifuge tube and add 500 µL of chloroform.

viii. Mix by inverting the sample several times and centrifuge at 1700 x g.

ix. Add 800 µL of ice cold ethanol and incubate at -20°C for one hour to precipitate the DNA.

x. Centrifuge at 12,000 x g for 10 minutes at 4°C, decant the supernate, and resuspend the pellet in 50 µL of TE buffer (10mM Tris, 0.1 mM EDTA, pH 8.0).

xi. Incubate sample overnight at room temperature.

c. Quantification of DNA
It is advisable that extracted products be measured using a spectrophotometer to ensure that enough DNA was successfully extracted. Quantification guidelines are in 6.2.C.4 "Quantification of DNA."

Note: Quantify DNA of a representative sample (5% or 6%) from each group of a particular size range and assume all those within that size range have a similar concentration. If they do not range too widely, average the values and determine the DNA concentration. Then add an appropriate volume to each PCR assay such that the amount per reaction is between 100 and 300 ng.

d. Amplification of *M. cerebralis* DNA

i. Following general PCR protocols (6.2 PCR Quality Assurance/Quality Control). The reagents and primers for this reaction are:

1° Master Mix: 50 µL total reaction volume

H_2O – sterile, molecular biology grade	32.1 µL
Mg Cl_2 (50 mM)(5.6.K)	1.5 µL (final concentration 1.5 mM)
10X Taq Polymerase Buffer	5 µL
dNTPs (5 mM stock)	4 µL (final concentration 0.4mM)
Tr5-16 Primer 1 (20 µM)	2 µL (final concentration 0.8 µM)
Tr3-16 Primer 2 (20 µM)	2 µL (final concentration 0.8 µM)
Taq Polymerase (5 units/µL)	0.4 µL (2 units per reaction)

Primers (Andree et al. 1998)
Tr5-16 = 5'-GCA TTG GTT TAC GCT GAT GTA GCG A-3'
Tr3-16 = 5' -GAA TCG CCG AAA CAA TCA TCG AGC TA-3'

ii. Add PCR reagents **except for sample DNA** to the MM tube, adding water first and Taq last. Keep all reagents cold in frozen cryo-rack during mixing, and return them to freezer immediately after use.

iii. Place 48 µL of MM into each 0.5 mL PCR tube. Close caps tightly. Move PCR tubes to sample loading area.

iv. Load 2 µL of each sample DNA to the appropriately labeled PCR tubes.

v. Thermocycler should be programmed for 35 cycles of the following regime:

1. Cycle Parameters:

a. Denature at 95 °C for five minutes.

b. Then, 35 cycles at:
95 °C for one minute.
65 °C for 2.5 minutes.
72 °C for 1.5 minutes.

Note: For maximum sensitivity with weak positive samples these conditions are critical.

vi. For the nested amplification prepare the 2nd MM as before, but substitute primers Tr5-17 and Tr3-17. Transfer 1 µL of the amplification product from the first amplification to the appropriately labeled reaction tube containing the 2nd MM and load the thermal cycler using the same program as for the first amplification.

Primers (Andree et al. 1998)
Tr5-17 = 5’ -GCC CTA TTA ACT AGT TGG TAG TAT AGA AGC-3’
Tr3-17 = 5’-GGC ACA CTA CTC CAA CAC TGA ATT TG-3’

e. Visualization of PCR Product by Electrophoresis

   i. Prepare agarose gel as indicated in 6.3.C “Detection of Product” and load 6.0 µL of amplified product + 1.5 µL loading buffer into sample wells.

   ii. After electrophoresis of products (6.3.D “Electrophoresis”), stain and photograph gel as described in 6.3.E “Staining the Gel” through 6.3.G “Visualize the DNA.”

   iii. Carefully record locations of bands on positive control samples in relation to DNA ladder bands. **Detection of the expected 415 bp (base pair) amplicon in a sample amplified using the nested PCR protocol will result in corroboration of the presence of *M. cerebralis* in the sample. Any samples not yielding this band with no apparent assay problems is reported as negative.**

   iv. If any unusual bands are present in the samples or if bands are present in negative controls, re-run PCR reaction from template DNA tube.

   v. **Photo document all gels** and attach the photo to the case history information.

5.3 *Ceratomyxa shasta* (Ceratomyxosis)

Ceratomyxa shasta is difficult to detect because the life cycle of the parasite includes two alternate hosts: salmonids and the freshwater polychaete worm, *Manayunkia speciosa* (Bartholomew et al. 1997). Salmonids are the only known fish hosts for *C. shasta.* Although susceptibility may vary between species and strain, all salmonids should be considered susceptible. The parasite is enzootic in a number of waters in California, Oregon, Washington, Idaho, Alaska, and B.C., Canada (Bartholomew et al. 1989a), but has not been reported outside of that region. Diagnosis is complicated by the long period required for development of mature myxospores in the fish and by the pleomorphic appearance of the presporogonic stages. Parasite development is temperature dependent, but in general, at 12ºC, a minimum of 40 days is required for spore development in rainbow trout in the laboratory. Confirmation of *C. shasta* is based on identification of myxospores with the appropriate morphology or by PCR confirmation of presumptive presporogonic life stages.

A. Screening Test

1. **Examination of Wet Mounts** (Bartholomew et al. 1989a, Bartholomew 2001)

    a. To prepare a wet mount, the intestine is removed, placed on a disposable surface and opened longitudinally with a clean scalpel or scissors. Scrape the posterior 1/3 of the intestinal mucosa and mix in a drop of water on a microscope slide. Prepare wet mounts from any areas of hemorrhage as well.

    b. Scan wet mount in a systematic manner under phase contrast or bright field microscopy at 200 to 440X magnification. Examine the entire smear or an area equal to that under a 22 mm^2 coverslip.

    c. Presumptive diagnosis is based on identification of multicellular myxosporidian pre-sporogonic stages (trophozoites) (Figure 5.4A). Visualization of prespore stages is not sufficient for definitive diagnosis. Any samples in which the organisms are not seen may be discarded and reported as negative.

B. Confirmatory Test

Confirmation of *C. shasta* should rely on either detection of mature spores or on amplification of parasite DNA using the polymerase chain reaction (PCR).

1. **Morphology** (Bartholomew 2001)
   Confirmatory diagnosis of *C. shasta* is based on identification of the characteristic myxospore (Figure 5.2). Myxospores of *C. shasta* measure 14 to 23 µm long and 6 to 8 µm wide at the suture line. The ends of the spores are rounded and reflected posteriorly and the suture line is distinct.

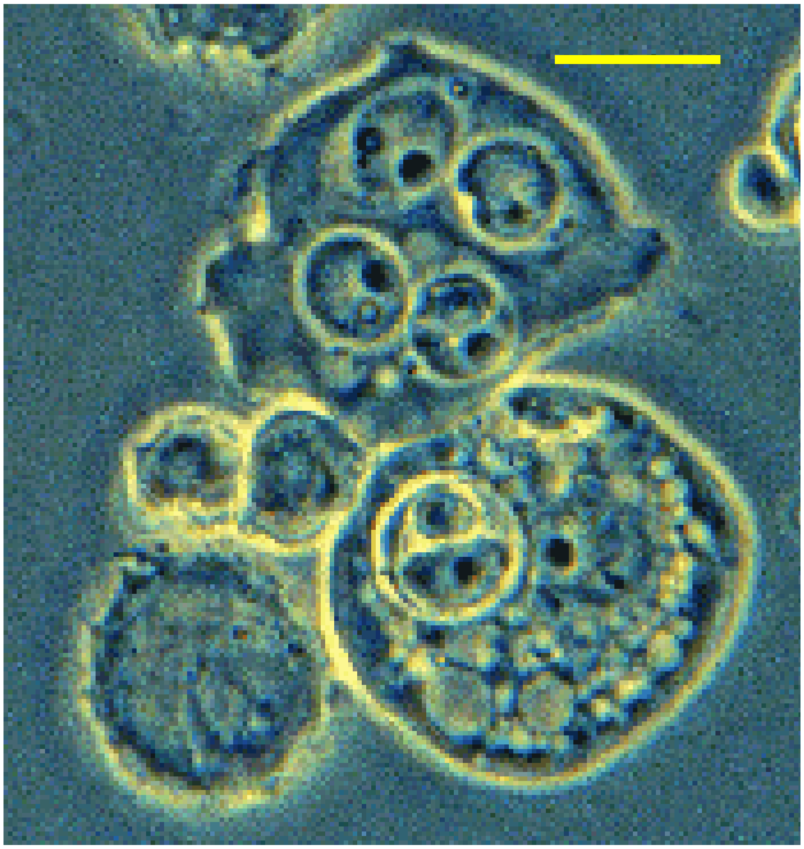


Figure 5.1. Wet mount of presporogonic stages of *Ceratomyxa shasta*. Bar equals 10 µm. Photograph courtesy of J. L. Bartholomew.

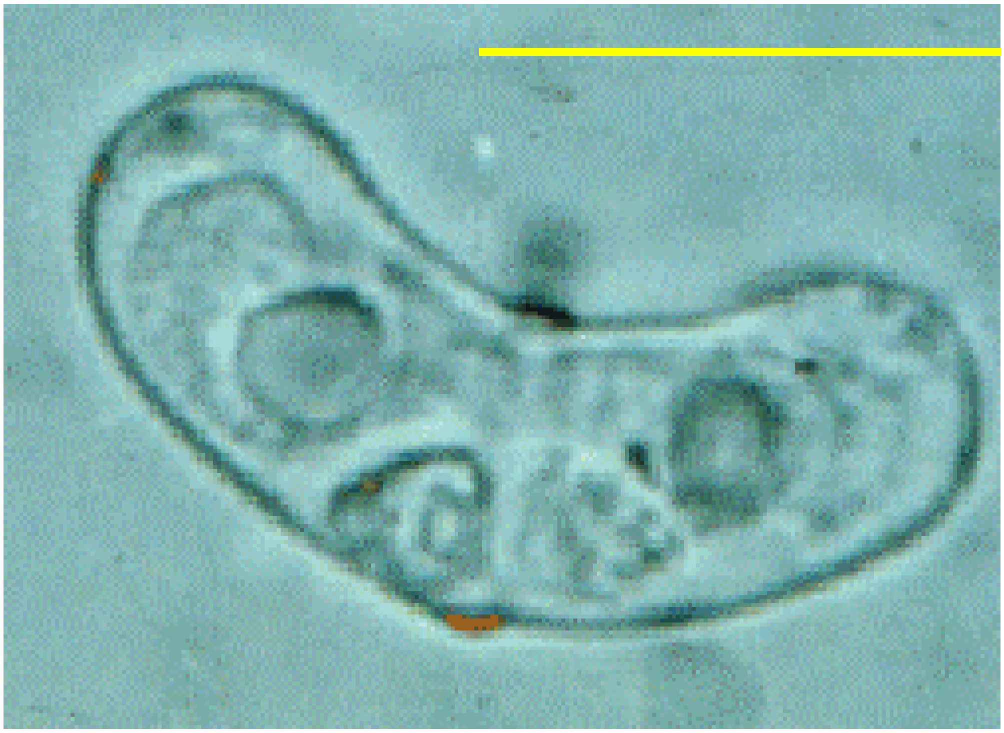


Figure 5.2. Wet mount of myxospore stage of *C. shasta***.** Bar equals 10 µm. Photograph courtesy of J. L. Bartholomew.

2. **Polymerase Chain Reaction** (Palenzuela et. al. 1999; Bartholomew 2001; Palenzuela and Bartholomew 2001)

   PCR should be utilized as a confirmatory test *only* on tissue collected at the time of inspection, using methods described under specimen collection.

   a. General Protocols and QA/QC Considerations (See 6.2 PCR Quality Assurance/Quality Control for specific QA/QC considerations and general protocols for PCR.)

   b. Extraction of DNA

      i. If the sample was fixed in ethanol, transfer to micro centrifuge tube with 500 µL DNA extraction buffer (5.6.G "DNA Extraction Buffer"); if frozen, add 500 µL DNA extraction buffer.

      ii. Add Proteinase K solution (5.6.E "Proteinase K") to a final concentration of 200 µg/mL (example: if stock solution is 20 mg/mL, add 5 µL).

      iii. Incubate micro centrifuge tubes at 37°C in a horizontal position on a slow platform rocker or with frequent inversion by hand. Digestion of sample will require about 4 to 5 hours, but overnight incubation does not affect the quality of the DNA and is recommended.

      iv. After samples are completely digested, add 5 µL of RNAse A (10 mg/mL stock) (5.6.F "RNAse A") to each micro centrifuge tube and incubate one hour at 37°C with rocking.

      v. Heat samples at 100°C for five minutes (in a water bath, heat block, or thermocycler).

      vi. Remove rack and cool at room temperature. Upon cooling, samples are ready for dilution and PCR. Samples at this stage may be stored at -20°C.

   c. Amplification of *C. shasta* DNA

      i. Dilute the DNA template (sample) 1:10 with ultra pure (molecular grade) sterile water.

         Note: Because this assay utilizes crude DNA, quantitation by UV spectrophometer does not provide useful information. A 1:10 dilution is generally sufficient unless the tissue size is larger than recommended (if this is suspected, include a 1:100 dilution in addition)

      ii. Following general PCR protocols (6.3 PCR Protocols), record appropriate data for PCR and calculate reagent volumes that go into the Master Mix (MM). This assay was optimized using reagents from Promega (Madison, WI, USA) and if other buffer systems are substituted, these should be tested. The primers and reagents for this reaction are:

Master Mix: 20 µL total reaction volume

H_2O – sterile, molecular biology grade	14.6 µL
Mg CL_2 (25 mM; Promega #A351B)	1.6 µL
10X PCR Poly Buffer (Promega #M190A)	2.0 µL
dNTPs (at 10mM each)	0.4 µL (final concentration 0.2 mM)
Forward Primer (100 µM)	0.1 µL (final concentration 0.5 µM)
Reverse Primer (100 µM)	0.1 µL (final concentration 0.5 µM)

Taq Polymerase (5 units/µL)	0.2 µL (1U per reaction)

Primers (Palenzuela and Bartholomew 2001):
Forward: 5' GGGCCTTAAAACCCAGTAG 3'
Reverse: 5' CCGTTTCAGGTTAGTTACTTG 3'

iii. Place 19 µL of MM into each 0.2 mL PCR tube. Close caps tightly. Move PCR tubes to sample loading area.

iv. Add 1 µL of each sample DNA to the appropriately labeled PCR tubes.

v. Thermocycler should be programmed for 35 cycles of the following regime:

1. Cycle Parameters:

a. Denature at 95°C for three minutes.

b. 35 Cycles of:
94°C for one minute.
58°C for 30 seconds.
72°C for one minute.
72°C for 10 minutes.
Hold to 4°C chill at end of program.

d. Detection

i. Prepare agarose gel as indicated in 6.3.C "Detection of Product" and load 10.0 µL of each PCR reaction + 1.5 µL loading buffer (5.6.H "Loading Buffer") into sample wells.

ii. After electrophoresis of products (6.3.D "Detection of Product"), stain and photograph gel as described in 6.3.E "Staining the Gel" through 6.3.G "Visualize the DNA."

iii. Carefully record locations of bands on positive control samples in relation to DNA ladder bands. ***C. shasta* positive reactions will have an amplicon of 640 bp. Any samples not yielding this band with no apparent assay problems is reported as negative and the samples are discarded.**

iv. If any unusual bands are present in the samples or if bands are present in negative controls, re-run PCR reaction from template DNA tube.

v. **Photo document all gels** (6.3.G "Visualize the DNA") and attach the photo to the case history information.

5.4 *Tetracapsula bryosalmonae* (Proliferative Kidney Disease)

The myxozoan (PKX), known to cause proliferative kidney disease in salmonids, is difficult to detect because its life cycle is complex and requires a bryozoan to complete its development (Canning et al. 1999). Diagnosis of infections in fish is also complicated because the parasite does not develop completely in the fish, and resulting myxospores, if present, lack fully developed spore valves (Kent 1994). Two developmental stages occur in salmonid hosts: presporogonic stages found in blood and interstitial kidney tissue and sporogonic stages found within the lumen of kidney tubules. Although PKX-like cells have been identified from species other than in the family Salmonidae, their identity as *T. bryosalmonae* is unconfirmed.

A. Screening Tests

1. **Leishman-Giemsa Staining** (Klontz and Chacko 1983)

    a. Stain slides with tissue imprints using Leishman-Giemsa stain.

        i The fixed imprint is incubated with approximately 1 mL Leishman stain (5.6.L "Leishman Stain") for one minute.

        ii Giemsa stain (2 to 3 mL) (5.6.M "Giemsa Stain") is added to the Leishman stain and allowed to stand for an additional 10 to 15 minutes.

        iii Rinse the slide and examine.

    b. Whenever possible, a positive control slide should be examined prior to evaluating samples.

    c. Examine a minimum of 100 fields of the stained imprints at 400x using bright field microscopy for the presporogonic stages of the parasite.

    d. Presporogonic stages (Figure 5.1) are large (approximately 20 µm) with a prominent cell membrane. The primary cell (outer, surrounding cell) contains prominent granules, a nucleus with a large nucleolus, and one or more secondary or daughter cells. Macrophages are frequently adhered to the surface of the parasite.

    e. **The finding of presporogonic stages (Figure 5.1) is presumptive and warrants confirmation by histology**.

2. **Lectin-Based Staining** (Hedrick et al. 1992)

    a. Tissue imprints are stained with 50 µL biotinylated GS-1 lectin (L-3759, Sigma, St. Louis, Missouri) suspended in 0.01M phosphate buffer pH 6.8 (5.6.I "0.01 M Phosphate Buffer pH 6.8," pH adjusted).

b. To determine the appropriate concentration of lectin, dilutions of 25 to 0.5 µg mL^{-1} are tested on control tissue.

c. Slides are incubated in a moist chamber for 1 to 2 hours at 25˚C.

d. Rinse three times in PBS (5.6.D “Phosphate Buffered Saline (PBS)”).

e. Apply 50 µL fluorescein avidin D (A-2001, Vector Laboratories, Inc., Burlingame, California) diluted to provide 10-30 µg mL^{-1} suspensions in PBS.

f. Incubate slides in moist chamber at 25˚C for 30 minutes.

g. Rinse three times, then carefully blot to near dry and mount with a coverslip using a drop of a mixture containing 1 part of 0.1M N-2-hydroxy-ethylpiperzine-N’-2-ethanesulfonic acid (HEPES) pH 8.0 and 9 parts glycerol.

h. Observe under a microscope with a UV light source equipped for fluorescein.

i. **The identification of fluorescing presporogonic stages (Figure 5.2) is presumptive and warrants corroboration by histology.**

B. Confirmatory Test

1. **Histopathology** (Hedrick et al. 1986)

a. Slides should be prepared from the kidney tissue that was placed in fixative at the time of collection using standard histological techniques.

b. Sections should be stained with hematoxylin and eosin (H&E) or Giemsa and examined for stages of *T. bryosalmonae*.

c. **The presence of presporogonic stages of *T. bryosalmonae* within the interstitial tissue of the kidney is corroboration of the infection (Figure 5.3).** A chronic inflammatory response is typically associated with the presence of the parasite (Figure 5.4) and a “whorled” appearance may be visualized corresponding to locations of the parasite. The presporogonic stage is approximately 20 µm in diameter and the primary cell has a lightly staining cytoplasm with a large, eosinophilic staining nucleolus (Figure 5.5). The primary cell may contain one to several spherical, dense, secondary (daughter) cells and macrophages are frequently seen adhered to the parasite. The inflammatory cell infiltrate is primarily composed of macrophages but numerous lymphocytes are also typically present. Sporogonic stages of *T. bryosalmonae* may be observed in the lumina of kidney tubules. Morphology of spores is poorly defined in fish and it appears that hardened valves typical of other myxosporidians do not form. Valveless myxospores are approximately 12 um X 7 um and have two spherical polar capsules 2 um in diameter (Figure 5.6).

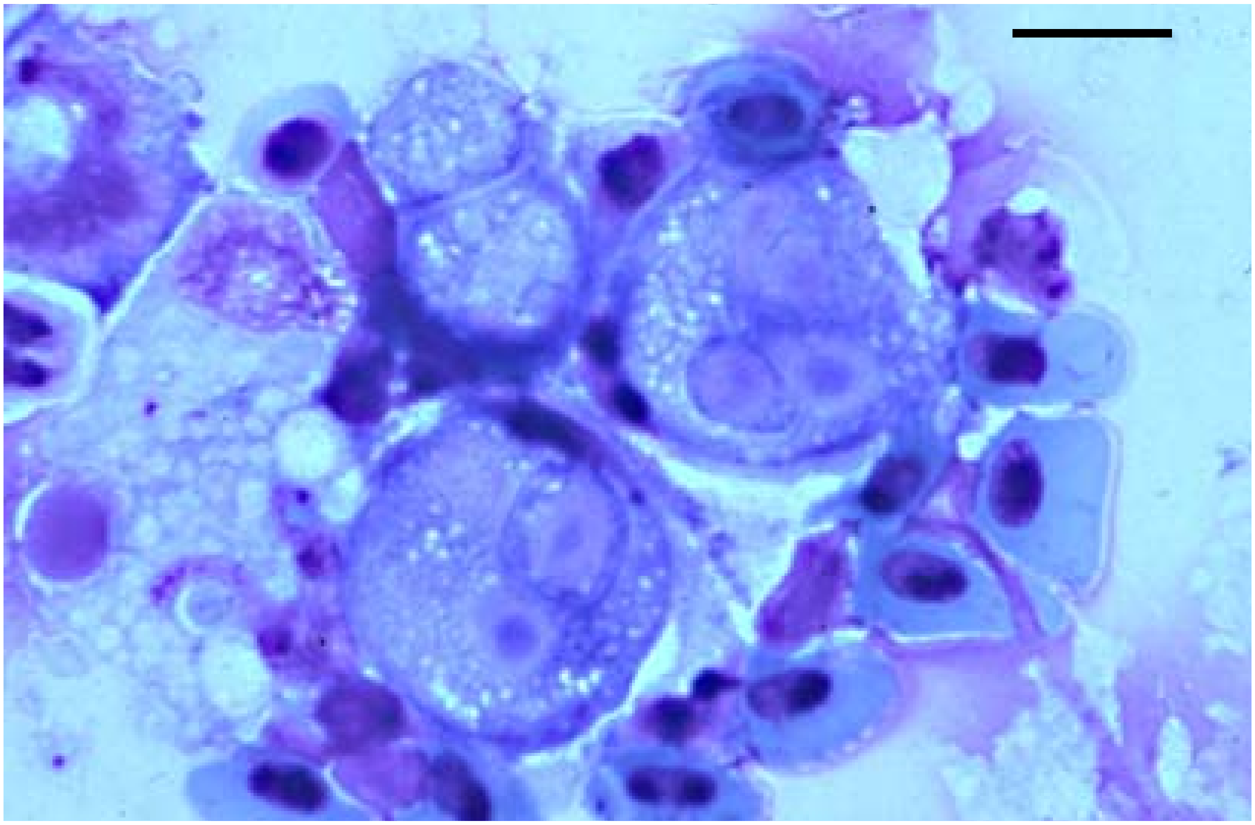


Figure 5.1. Multicellular *Tetracapsula bryosalmonae* in a Leishman-Giemsa stained imprint. Bar is 10 µm. Photograph courtesy of R. P. Hedrick.

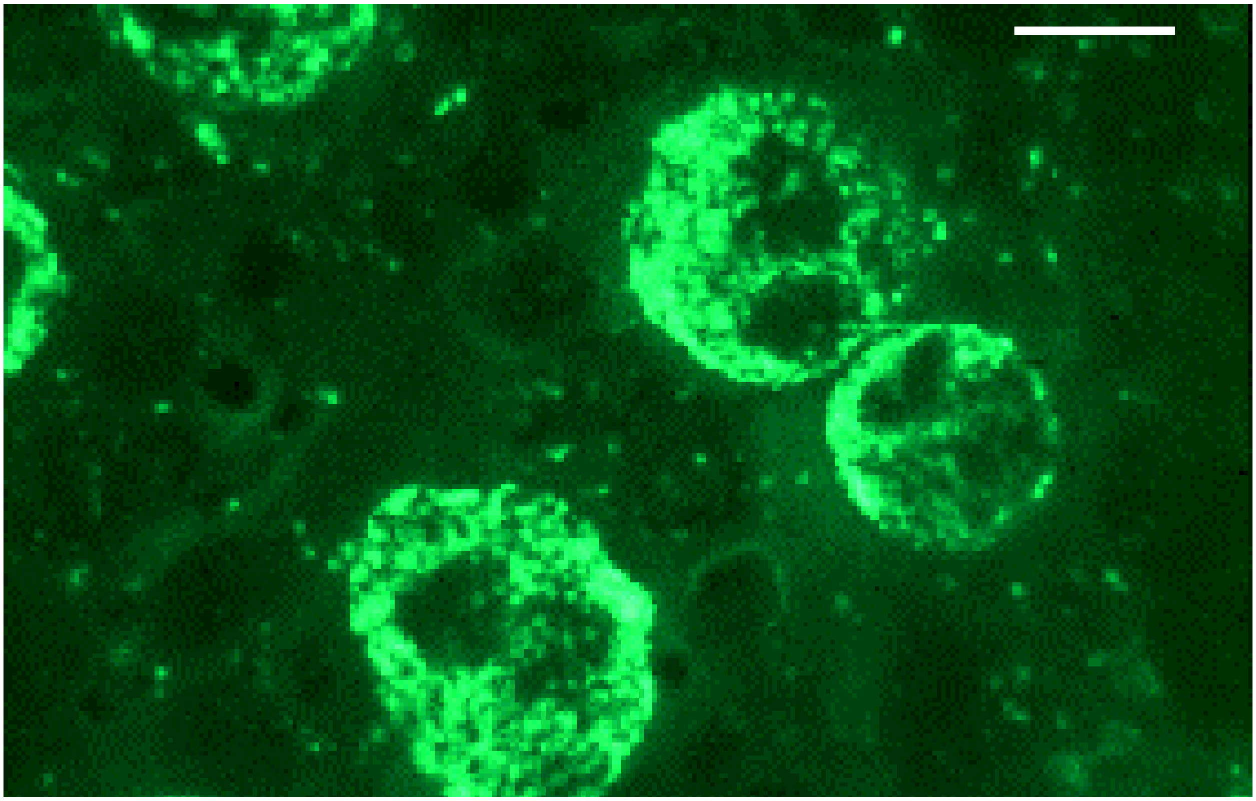


Figure 5.2. Multicellular *Tetracapsula bryosalmonae* in a lectin-stained imprint. Bar is 10 µm. Photograph courtesy of R. P. Hedrick.

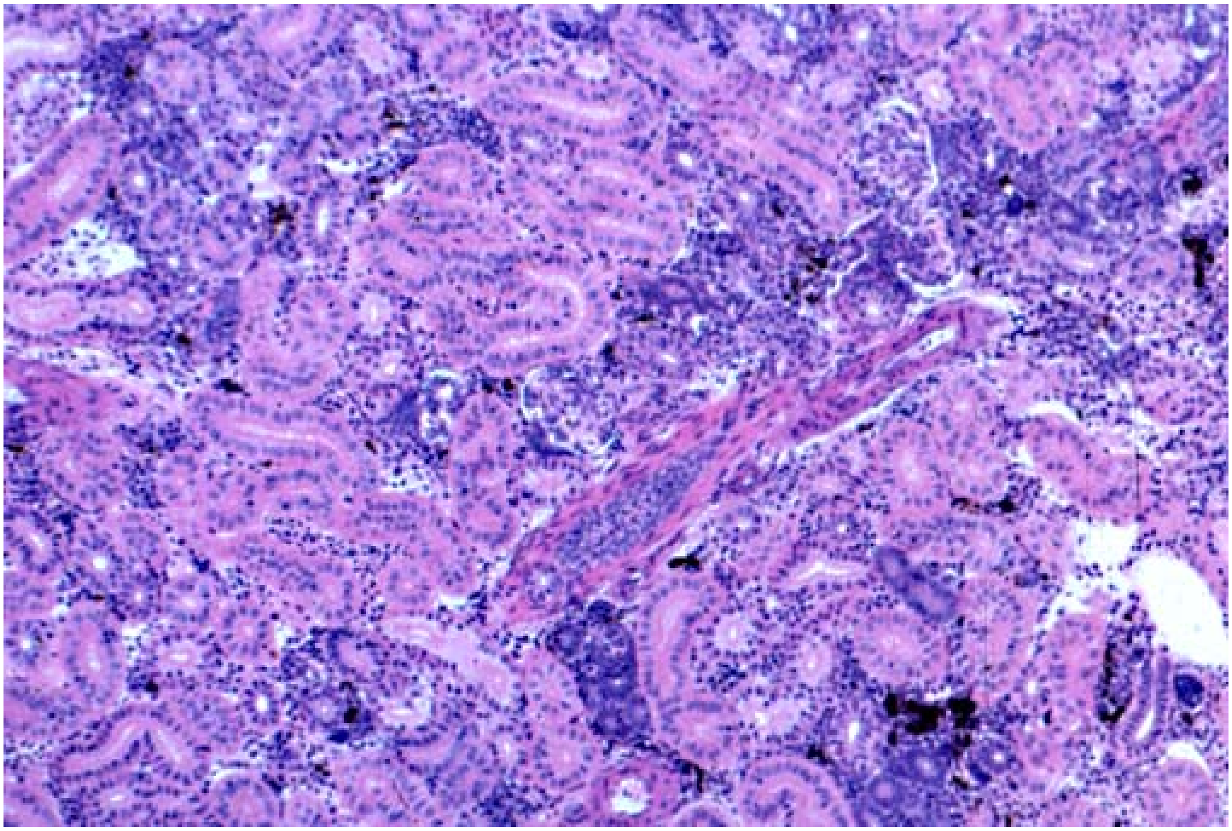


Figure 5.3. Histological sections of kidney tissue infected with *T. bryosalmonae* at low magnification of normal kidney. Photographs courtesy of R. P. Hedrick.

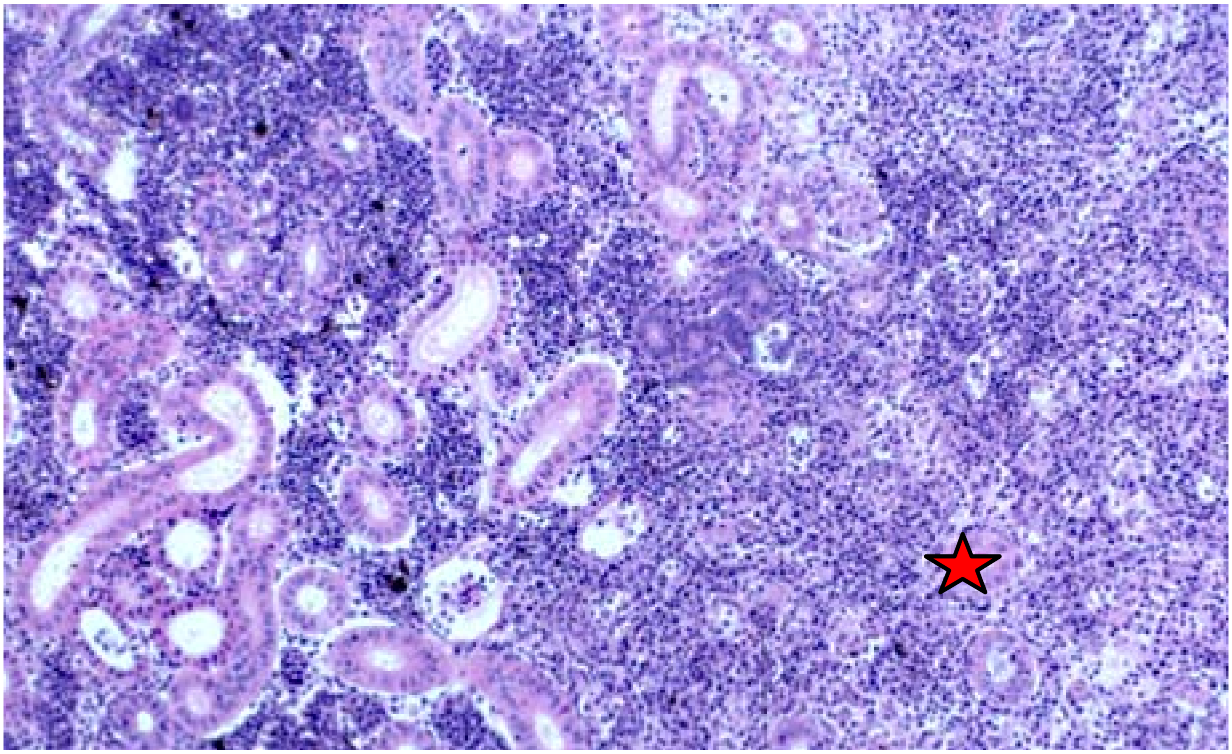


Figure 5.4. Histological sections of kidney tissue infected with *T. bryosalmonae*. at low magnification showing area of inflammatory cell infiltrate indicated by the star. Photographs courtesy of R. P. Hedrick.

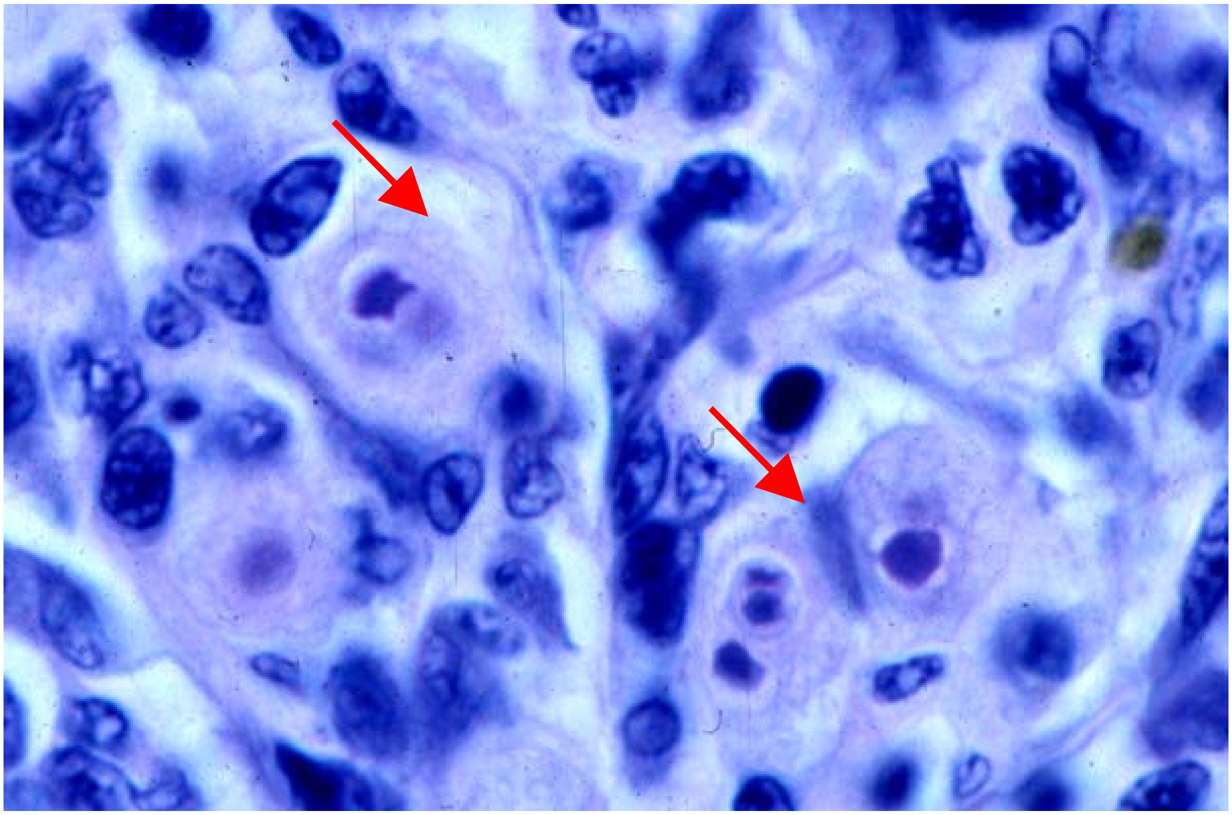


Figure 5.5. Histological sections of kidney tissue infected with *T. bryosalmonae*. at high (400X) magnification of parasites in tissue. Photographs courtesy of R. P. Hedrick.

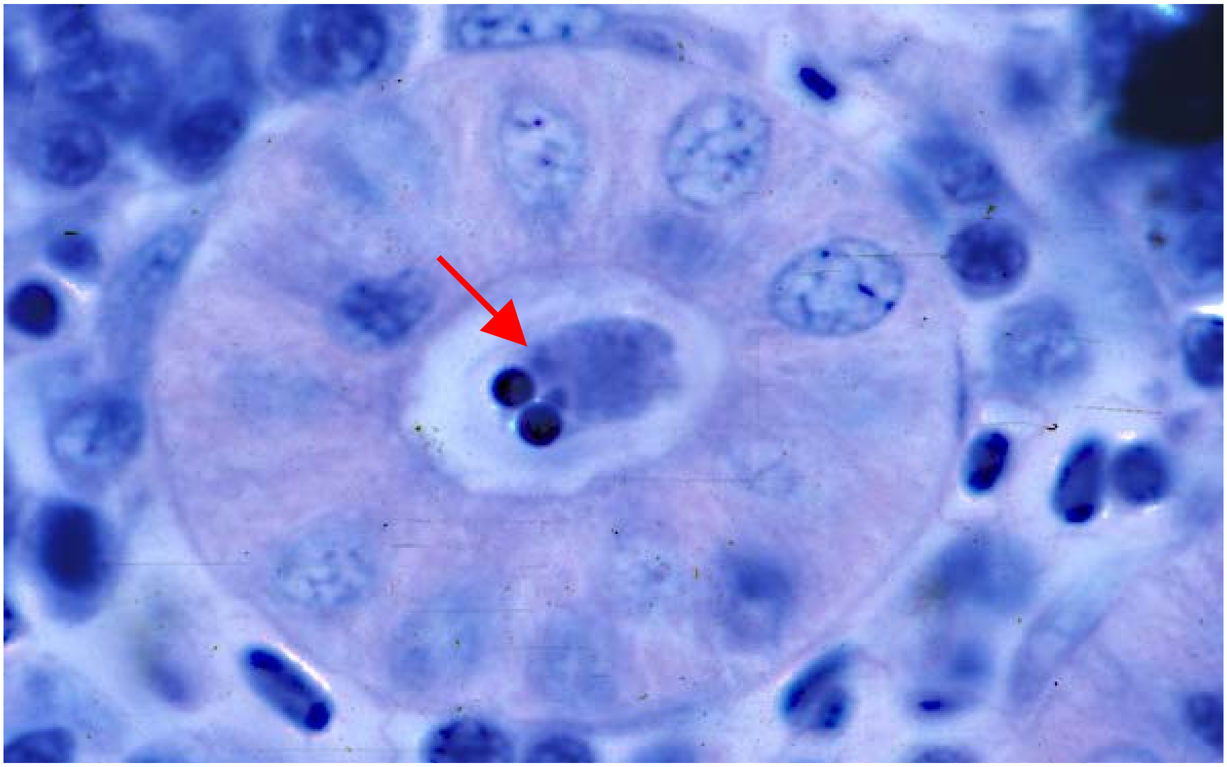


Figure 5.6. Histological sections of kidney tissue infected with *T. bryosalmonae*. sporogonic stage in kidney tubule (1000X). Photographs courtesy of R. P. Hedrick.

2. Polymerase Chain Reaction (Kent et al. 1998; Feist et al. 2001)

PCR should be utilized as a confirmatory test *only* on tissue collected at the time of inspection, using methods described under specimen collection.

a. General Protocols and QA/QC Considerations (See 6.2 PCR Quality Assurance/Quality Control for specific QA/QC considerations and general protocols for PCR.)

b. Extraction of DNA
Note: This assay was optimized using phenol:chloroform extraction and if other extraction methods are substituted, these should be tested.

i. If the sample was fixed in ethanol, wash twice in TE buffer (6.4 Reagents), then soak in TE buffer for one hour. Lyse sample in 500 µL DNA extraction buffer (5.6.G "DNA Extraction Buffer") with Proteinase K solution (5.6.E "Proteinase K") to a final concentration of 200 µg/mL (example: if stock solution is 20 mg/mL, add 5 µL) with agitation for at least one hour at 60˚C, or overnight at 37˚C.

ii. Extract twice with 400 µL phenol/chloroform/isoamy alcohol (50:50:1).

iii. Precipitate DNA in 800 µL absolute ethanol and 40 µL 3 µ sodium acetate, centrifuge at 13000 x g for 15 minutes and remove ethanol. Rinse with 70% ethanol, centrifuge at 13000 x g for 10 minutes and remove ethanol. Air dry pellet and resuspend in 20 µL molecular grade water. Sample can be stored at -20˚C.

c. Quantitation of DNA
It is advisable that extracted products be measured using a spectrophotometer to ensure that enough DNA was successfully extracted. Quantification guidelines are in 6.2.C "Extraction of DNA or RNA from Samples."

Note: Quantify DNA of a representative sample (5% or 6%) from each group of a particular size range and assume all those within that size range have a similar concentration. If they do not range too widely, average the values and determine the DNA concentration. Then add an appropriate volume to each PCR assay such that the amount per reaction is between 100 and 300 ng.

d. Amplification of *T. bryosalmonae* DNA

i. Following general PCR protocols (6.3 PCR Protocols), record appropriate data for PCR and calculate reagent volumes that go into the Master Mix (MM). This assay was optimized using reagents from GIBCO (Gaithersburg, MD, USA) and if other buffer systems are substituted, these should be tested. The primers and reagents for this reaction are:

Master Mix: 50 µL total reaction volume

H_2O – sterile, molecular biology grade	33.4 µL
Mg CL_2 (25 mM)	5.0 µL (final concentration 2.5 mM)
10X PCR buffer	5.0 µL
dNTPs (25mM)	0.4 µL (final concentration 0.2 mM)
5-Forward primer (20 µM)	2.0 µL (final concentration 0.8 µM)
6-Reverse primer (20 µM)	2.0 µL (final concentration 0.8 µM)

Taq polymerase (5 units/µL)	0.2 µL (1U per reaction)

Primers (Kent et al. 1998):
Forward (5F): 5' CCTATTCATTGAGTAGAGA 3'
Reverse (6R): 5' GGACCTTACTCGTTTCCGACC 3'

ii. Place 48 µL of MM into each 0.5 mL PCR tube. Close caps tightly. Move PCR tubes to sample loading area.

iii. Add 2 µL of each sample DNA to the appropriately labeled PCR tubes.

iv. Thermocycler should be programmed for 35 cycles of the following regime:

1. Cycle Parameters:
Denature at 94°C for three minutes.

2. 35 Cycles of:

94°C for one minute.
55°C for one minute.
72°C for one minute.
72°C for five minutes.
Hold to 4°C chill at end of program.

e. Detection

i. Prepare agarose gel as indicated in 6.3.C "Detection of Product" and load 10.0 µL of each PCR reaction + 1.5 µL loading buffer (5.6.H "Loading Buffer") into sample wells.

ii. After electrophoresis of products (6.3.D "Electrophoresis"), stain and photograph gel as described in 6.3.E "Staining the Gel" through 6.3.G "Visualize the DNA."

iii. Carefully record locations of bands on positive control samples in relation to DNA ladder bands. *T. bryosalmonae* positive reactions will have an amplicon of 501 bp. Any samples not yielding this band with no apparent assay problems is reported as negative and the samples are discarded.

iv. If any unusual bands are present in the samples or if bands are present in negative controls, re-run PCR reaction from template DNA tube.

v. **Photo document all gels** (6.3.G "Visualize the DNA") and attach the photo to the case history information.

5.5 *Bothriocephalus acheilognathi* (Bothriocephalosis, Asian Tapeworm)

Bothriocephalosis is an intestinal infection of certain fish by the cestode *Bothriocephalus acheilognathi* (Mitchell 1994; Scholtz 1997), a Pseudophyllidian tapeworm. The infecting organism is also known as the Asian or Asian fish tapeworm and as the Chinese tapeworm. The Asian tapeworm has been reported in Asia, Europe, Australia, South Africa, and North America. In North America, it has been reported in Mexico, British Columbia, throughout the southern half of the United States, and in New Hampshire, New York, and Hawaii. Fish become infected after ingesting infected copepods and development of the worm occurs in the anterior intestinal tract. *Bothriocephalus acheilognathi* is a thermophile that has an optimum temperature for growth and maturation above 25ºC.

Most members of the Family Cyprinidae are considered potential hosts, with the exception of goldfish, *Carassius auratus*. Infections have also been reported in species from the following families: Siluridae, Poeciliidae, Percidae, Centrarchidae, Gobiidae, and Cyprinodontidae

A. Screening Test

1. **For Fish Less than 20 cm in Length**
   The uncoiled intestines from several fish may be placed side by side on a microscope slide or glass plate (9 x 9 x 0.3 cm). When intestines are too small to uncoil they can be placed as they come onto a slide. There is no need to slit the intestines. A second slide or glass plate placed over the excised intestine spreads the intestine for easy visibility. For small intestines the weight of the glass plates is usually sufficient to flatten the contents for easy viewing. Binder clips can be used if further flattening is desired (Mitchell 1989, 1994).

   a. Examination requires the use of a 15 to 30-power dissecting microscope. Reflected light gives the best results.

   b. *Bothriocephalus acheilognathi* sometimes takes on a silvery cast and movement will be detected if the specimen is viewed for 15 seconds.

   c. Thoroughly examine the anterior intestinal tract. Small worms may measure only 350 μm in length.

2. **For Fish Longer than 20 cm**
   Slit the anterior third of the gut open lengthwise and carefully remove any cestodes while keeping the scolex intact. Scraping the inner wall of the intestine with a scalpel ensures the collection of the scolices from visible worms and from small worms not seen. Place contents on a glass plate (see above) or microscope slide (depending on volume of contents) and cover with another plate or slide. Using binder clips, press the two plates together. Examine entire sample for *B. acheilognathi*.

3. **Visualization of a cestode found in the anterior third of the intestine that forms a pyramidal scolex in the semi contracted state (Figure 5.1) is a presumptive positive classification**. In the semi contracted state the scolex is usually three times the width of the segmented portion adjacent to the scolex. If large worms or many small worms are present, they may be apparent as a yellow to white bulge in the intestine.

B. Confirmatory Test

Other cestodes have similar pyramid shaped scolices. Therefore, a key must be used to definitively identify *B. acheilognathi*. A complete key for the identification of *B. acheilognathi* and accompanying figures are found in the 4th Edition of the Blue Book (Thoesen 1994). Specific characteristics needed for definitive identification are noted below.

1. ***Bothriocephalus acheilognathi***
   Is a complete and distinctly segmented, thin tapeworm that can reach a length of over 50 cm, but is usually less than 10 cm. Segmentation is evident on worms 1 mm or more in length (Figure 5.1).

2. ***Bothriocephalus acheilognathi***
   Has a flattened scolex with two bothria (deep, elongated sucking grooves dorsal and ventral as seen in Figure 5.1, no hooks, no spines, no true suckers, and no proboscides (short tentacles). In the lateral view, the scolex takes a strong arrowhead appearance when semi contracted and a balled or squared appearance when fully contracted. The posterior portion of the scolex is wider than the first few segments in both the extended and contracted positions.

3. ***Bothriocephalus acheilognathi***
   Has no neck and no dorsal or ventral median furrow.

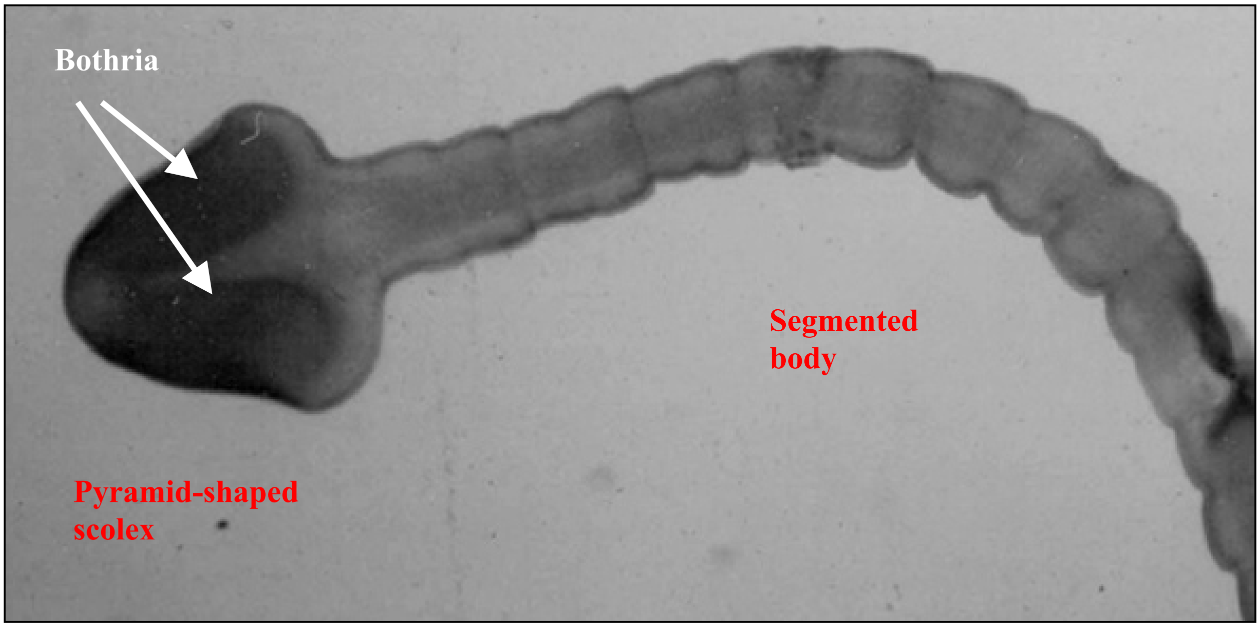


Figure 5.1. *Bothriocephalus acheilognathi* showing a pyramid shaped scolex, segmentation, and two bothria. Photograph courtesy of A. Mitchell.

5.6 Media and Reagents

A. 0.5% Pepsin Solution

Pepsin	5.0 g
HCl	5.0 mL
Sterile distilled water	to 1000 mL

Store at 4°C.

B. 0.5% Trypsin Solution

EDTA	0.2 g
NaCl	8.0 g
KCl	0.2 g
KH2PO4	0.2 g
NaHPO4	1.15 g
Trypsin	5.0 g
Sterile distilled water	to 1000 mL

Store at 4°C.

C. 1N Sodium Hydroxide (NaOH)

NaOH	40.0 g
Distilled water	to 1000 mL

D. Phosphate Buffered Saline (PBS)

Sodium Chloride (NaCl)	8.0 g
Monopotassium phosphate (KH_2PO_4)	0.34 g
Dipotassium phosphate (K_2HPO_4)	1.22 g
Distilled water	to 1000 mL

Filter with 0.22 um filter. Store at room temperature.

E. Proteinase K

Can be obtained as a stable liquid solution (14 to 22 $mg.mL^{-1}$) from commercial sources. If kept at 4°C, it is stable for > one year.

F. RNAse A

It is a 10 mg.mL^{-1} solution and can be obtained from commercial sources [e.g. 5'-3' (Cat. # is 5-888777)] as a 50% glycerol solution that is liquid at -20°C.

G. DNA Extraction Buffer

The buffer is NaCl 100 mM, Tris-HCl 10 mM, EDTA 25 mM, SDS 1%. The stock solutions are:

NaCl 5M	{50X}
Tris-HCl 1M, pH 7.8	{100X}
EDTA 0.5M, pH 8	{20X}
SDS 20%	{20X}

Stock solutions should be made using ultrapure, nuclease-free water (HPLC grade or equivalent), aliquoted and stored at -20°C until needed. Pre-made stock solutions (molecular biology grade) can be purchased from a commercial supplier, aliquoted and frozen, so the chances of contamination are reduced.

H. Loading Buffer

Sigma P-7206 Pre-made 6X concentrate, ready to use (store -20°C)

OR

Bromophenol blue	0.25%
Xylene cyanol	0.25%
Glycerol	30.0%

I. 0.01 M Phosphate Buffer pH 6.8

Adjust pH of PBS recipe above to 6.8.

J. 10% Neutral Buffered Formalin

Formalin, concentrated	100 mL
Distilled water	900 mL
Sodium phosphate (monobasic)	4.0 g
Sodium phosphate (dibasic)	6.5 g

K. 2.5 mM Magnesium Chloride ($MgCl_2$)

It can be obtained from commercial sources and often is supplied with Taq enzyme. Purchase as molecular biology grade.

L. Leishman Stain

Purchase from commercial source.

M. Giemsa Stain

Stock solution:

Giemsa powder	1 g
Glycerin	66 mL

Mix and place in oven at 56 to 66˚C for 0.5 to 1 hour. Add 66 mL absolute methanol after solution has cooled.

Phosphate buffer, pH 6.0

Sodium phosphate (monobasic)	35 g
Sodium phosphate (dibasic)	4.84 g
Distilled water	to 4 L

Working Giemsa

Stock Giemsa	14 mL
Phosphate buffer	200 mL

Must be made fresh.

5.7 Glossary

A. Myxozoan Life Stages

Trophozoite - vegetative, uninucleate stage with a single diploid nucleus.

Presporogonic stages - multicellular parasite stages that will eventually give rise to spores. For purposes here, this encompasses stages referred to as plasmodia, sporoblasts and sporocysts.

Spore - myxospore is the mature spore that develops in the fish. It contains polar capsules, polar filaments and sporoplasm cell(s).

Sporogenesis - the process of spore formation.

B. Cestode Morphology

Scolex - portion of a cestode that bears the organs of attachment.

Bothria - organ of attachment; characteristically they are long narrow grooves of weak muscularity; may become flattened to form an efficient sucking organ.

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